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Quantitative Analysis of Steroidal Glycosides in Different Organs of Easter Lily (*Lilium longiflorum* Thunb.) by LC-MS/MS

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The bulbs of the Easter lily (*Lilium longiflorum* Thunb.) are regularly consumed in Asia as both food and medicine, and the beautiful white flowers are appreciated worldwide as an attractive ornamental. The Easter lily is a rich source of steroidal glycosides, a group of compounds that may be responsible for some of the traditional medicinal uses of lilies. Since the appearance of recent reports on the role steroidal glycosides in animal and human health, there is increasing interest in the concentration of these natural products in plant-derived foods. A LC-MS/MS method performed in multiple reaction monitoring (MRM) mode was used for the quantitative analysis of two steroidal glycoalkaloids and three furostanol saponins, in the different organs of *L. longiflorum*. The highest concentrations of the total five steroidal glycosides were 12.02 ± 0.36, 10.09 ± 0.23, and 9.36 ± 0.27 mg/g dry weight in flower buds, lower stems, and leaves, respectively. The highest concentrations of the two steroidal glycoalkaloids were 8.49 ± 0.3, 6.91 ± 0.22, and 5.83 ± 0.15 mg/g dry weight in flower buds, leaves, and bulbs, respectively. In contrast, the highest concentrations of the three furostanol saponins were 4.87 ± 0.13, 4.37 ± 0.07, and 3.53 ± 0.06 mg/g dry weight in lower stems, fleshy roots, and flower buds, respectively. The steroidal glycoalkaloids were detected in higher concentrations as compared to the furostanol saponins in all of the plant organs except the roots. The ratio of the steroidal glycoalkaloids to furostanol saponins was higher in the plant organs exposed to light and decreased in proportion from the aboveground organs to the underground organs. Additionally, histological staining of bulb scales revealed differential furostanol accumulation in the basal plate, bulb scale epidermal cells, and vascular bundles, with little or no staining in the mesophyll of the bulb scale. An understanding of the distribution of steroidal glycosides in the different organs of *L. longiflorum* is the first step in developing insight into the role these compounds play in plant biology and chemical ecology and aids in the development of extraction and purification methodologies for food, health, and industrial applications. In the present study, (22*R*,25*R*)-spirosol-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside, (22*R*,25*R*)-spirosol-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-[6-*O*-acetyl-β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside, (25*R*)-26-*O*-(β-D-glucopyranosyl)furost-5-ene-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside, (25*R*)-26-*O*-(β-D-glucopyranosyl)-furost-5-ene-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→3)-β-D-glucopyranoside, and (25*R*)-26-*O*-(β-D-glucopyranosyl)furost-5-ene-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-xylopyranosyl-(1→3)-β-D-glucopyranoside were quantified in the different organs of *L. longiflorum* for the first time.

KEYWORDS: *Lilium longiflorum* Thunb.; Liliaceae; Easter lily; steroidal glycoside; steroidal glycoalkaloid; furostanol saponin

INTRODUCTION

The Easter lily (*Lilium longiflorum* Thunb., family Liliaceae), with its showy white flowers and fragrant aroma, is enjoyed worldwide as an attractive ornamental plant. Easter lilies are most commonly seen as indoor potted plants or floral arrangements around the Easter holidays; however, they are also often planted outdoors as bedding plants in flower gardens. In addition to their esthetic value, lily bulbs and flower buds are regularly consumed as a food in Asia for their distinctive bitter taste and have a long historical use in traditional Chinese medicine. In particular, a preparation of bulbs of various *Lilium* species, referred to as "Bai-he", is used as a treatment for inflammation and lung ailments (*1*, *2*). Among many other secondary metabolites, *L. longiflorum* is a rich source of steroidal glycosides, a structurally diverse class of natural products that includes steroidal saponins and steroidal glycoalkaloids.

Steroidal glycosides have been reported to exhibit a wide range of biological activities including antifungal (*3*, *4*), platelet aggregation inhibition (*5*, *6*), anticholinergic (*7*),

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antidiabetic (*8*), antihypertensive (*9*), cholesterol lowering (*10*), anti-inflammatory (*11*), antiviral (*12*), and anticancer (*13*−*16*). Additionally, steroidal glycosides have a wide variety of commercial uses including as surfactants (*17*), foaming agents (*18*), and vaccine adjuvants (*19*) and serve as precursors for the industrial production of pharmaceutical steroids (*20*).

Steroidal saponins have been found in over 100 plant families and in some marine organisms such as starfish and sea cucumber (*21*). They are characterized by a steroid type skeleton glycosidically linked to carbohydrate moieties. Steroidal glycoalkaloids are characterized by a nitrogen-containing steroid type skeleton glycosidically linked to carbohydrate moieties. In contrast to steroidal saponins, the occurrences of steroidal glycoalkaloids are, thus far, limited to members of the plant families Solanaceae and Liliaceae (*22*, *23*). Some glycoalkaloids from solanaceaous plants have been shown to play a role in plant defense and are toxic to animals and humans. The potato glycoalkaloids, α-solanine and α-chaconine, are highly toxic to animals due to their interaction with membrane sterols, disruption of cell membranes, and inhibition of acetylcholinesterase, suggesting a biological role in antiherbivory (*24*). In *Lilium*, steroidal glycoalkaloids have been identified in *L. philippinense* Baker (*25*), *L. candidum* L. (*26*), and *L. brownii* var. *viridulum* (*27*), and recently solasodine-based glycoalkaloids were identified for the first time from *L. longiflorum* (*28*). Interestingly, both the leaves and flowers of *L. longiflorum* have been reported to be highly nephrotoxic to domesticated cats; however, the toxic compounds have yet to be identified (*29*, *30*). Although it has been reported that solasodine-based glycoalkaloids are less toxic then solanidine-based glycoalkaloids (*31*), the animal and human toxicity of the steroidal glycoalkaloids from *L. longiflorum* has yet to be investigated.

Although the putative biological activities of steroidal glycosides are well documented, the biological role of these compounds in plant metabolism and development is poorly understood. The role of steroidal glycosides in wound response and plant defense, including antifungal and antiherbivory, has been studied extensively (*32*−*45*). In fact, some steroidal glycosides are toxic to insects such as the European corn borer, *Ostrinia nubialis* (*33*), and army worm, *Spodoptera littoralis* (*34*). In oats, *Avena sativa*, biologically inactive steroidal saponins are converted into an antifungal form in response to tissue damage, suggesting a role in the plant−pathogen interaction (*36*, *40*, *43*, *44*). In addition, the steroidal glycoalkaloids α-tomatine and α-chaconine play a role in fungal resistance of tomato, *Solanum lycopersicum*, and potato, *Solanum tuberosum*, respectively (*37*). Although the literature suggests that steroidal glycosides are involved in the plant−pathogen interaction and antiherbivory in oats, tomato, and potato, there are no reports on biological role of steroidal glycosides in *L. longiflorum*.

Recently, two steroidal glycoalkaloids, (22*R*,25*R*)-spirosol-5-en-3*β*-yl *O*-α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranoside (**1**) and (22*R*,25*R*)-spirosol-5-en-3*β*-yl *O*-α-L-rhamnopyranosyl-(1→2)-[6-*O*-acetyl-*β*-D-glucopyranosyl-(1→4)]-*β*-D-glucopyranoside (**2**), and three furostanol saponins, (25*R*)-26-*O*-(*β*-D-glucopyranosyl)furost-5-ene-3*β*,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranoside (**3**), (25*R*)-26-*O*-(*β*-D-glucopyranosyl)furost-5-ene-3*β*,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→3)-*β*-D-glucopyranoside (**4**), and (25*R*)-26-*O*-(*β*-D-glucopyranosyl)furost-5-ene-3*β*,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-xylopyranosyl-(1→3)-*β*-D-glucopyranoside (**5**), were identified in *L. longiflorum* (*28*). Although steroidal glycosides have attracted scientific attention in recent years for their medicinal and industrial uses, there are only a few studies that have quantified these compounds within different plant organs throughout plant development. The organ distribution of steroidal glycosides in various plants such as *Solanum nigrum*, *Solanum incanun* (*46*), *Asparagus officinalis* L. (*47*), and *Dioscorea pseudojaponica* (*48*) has been reported; however, there are no studies on the distribution of steroidal glycosides in *L. longiflorum*.

Steroidal glycosides lack a strong chromophore and occur in complex biological matrices; therefore, nonspecific short-wavelength UV detection is often inadequate. Analytical methods using evaporative light scattering detection (ELSD) are used to help overcome this obstacle; however, laborious sample preparation and sensitivity issues persist (*49*). LC-MS methods operating in selected ion monitoring (SIM) mode have been developed to increase sensitivity and specificity; however, the separation of structurally similar compounds and shared ions still poses a challenge (*23*, *49*). LC-MS/MS in MRM mode overcomes these obstacles, allowing for sensitive quantitative analysis in complex biological matrices with increased specificity over SIM.

The purpose of this investigation was to utilize LC-MS/MS in MRM mode to quantify five steroidal glycosides in the different organs of *L. longiflorum*. Additionally, histological techniques were employed to qualitatively visualize tissue-specific localization of furostanols in bulb scales. An understanding of the distribution and tissue specific localization of steroidal glycosides in *L. longiflorum* is the first step to develop insight into the biological role these compounds play in plant metabolism, plant development, and chemical ecology. Quantitative analysis of steroidal glycosides in the different organs of *L. longiflorum* will aid in development studies in animal and human health, toxicology, and optimization of extraction methodologies for potential commercial applications including functional foods, cosmetics, and pharmaceuticals.

MATERIALS AND METHODS

Plant Material. Ten *L. longiflorum* cv. 7-8 plants were grown from tissue-cultured bulbs provided by the Rutgers University Easter lily breeding program. The young bulbs were treated with Captan (Bayer CropScience AG, Monheim am Rhein, Germany) fungicide prior to planting. Bulbs were planted in raised beds containing Pro-Mix (Premier Horticulture Inc., Quakertown, PA) soil mix and were grown to mature plants, containing both flower buds and flowers, under greenhouse conditions for 9 months prior to harvest. The greenhouse temperatures were set to provide a minimum day temperature of 24 °C and a minimum night temperature of 18 °C. Plants were fertilized biweekly with a 100 mg/L solution of NPK 15-15-15 fertilizer (J. R. Peters Inc., Allentown, PA). Each plant was harvested by hand and manually separated into the following plant organs: bulb scales, fibrous roots, fleshy roots, leaves, lower stems, upper stems, flower buds, and mature flowers. Bulb scales included both inner and outer bulb scales and ranged from 0.8 to 2.0 cm in width and from 0.9 to 4.0 cm in length. Fibrous roots were 0.25−0.5 mm in diameter. Fleshy roots were 2−4 mm in diameter. Leaves ranged in size from 6 to 14 cm. Lower stems were defined as the underground portion of the stem, ranged in size from 6 to 10 cm, and were from white to yellow in appearance. Upper stems were defined as the aboveground portion of the stem, ranged in size from 19 to 31 cm, and were green in appearance. Flower buds ranged in size from 3 to 6 cm. Mature flowers ranged in size from 6 to 14 cm. All of the organs from 10 individual plants were pooled together by organ type, immediately frozen under liquid nitrogen, lyophilized on a VirTis AdVantage laboratory freeze-dryer (SP Industries Inc., Warminster, PA), and stored at −80 °C until analyzed.

Chemicals. The following compounds were obtained commercially: *p*-(dimethylamino)benzaldehyde, hydrochloric acid, and pyridine-d_5 (0.3% v/v TMS) (Sigma-Aldrich, St. Louis, MO). All solvents (acetonitrile, chloroform, ethanol, ethyl acetate, formic acid, *n*-butanol, and *n*-pentane) were of chromatographic grade (Thermo Fisher Scientific Inc., Fair Lawn, NJ). Water was deionized (18 MΩ cm) using a Milli-Q water purification system (Millipore, Bedford, MA).

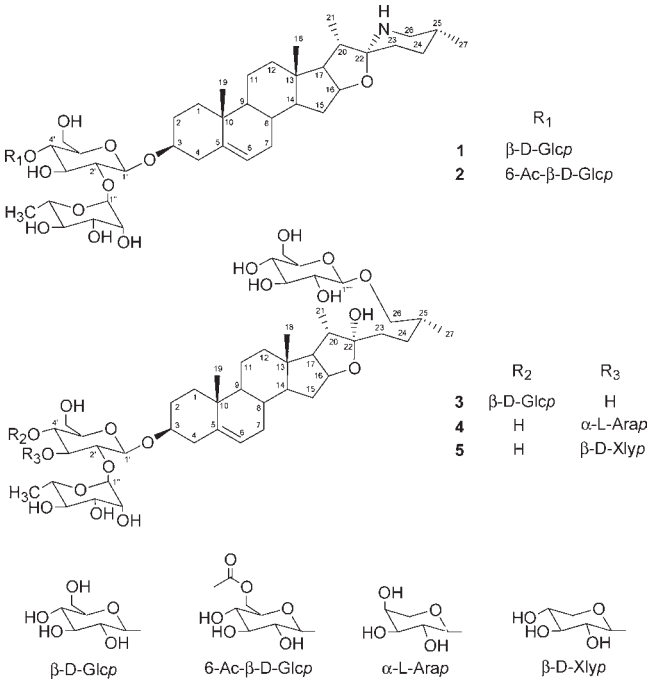

Figure 1. Structures of steroidal glycoalkaloids **1** and **2** and furostanol saponins **3**−**5** quantified in the various *L. longiflorum* organs.

Histology and Microscopy. Histological detection of furostanols was modified from the method of Gurielidze et al. (*50*). Bulb scales were carefully cross-sectioned (~0.5 mm) parallel to the basal plate, soaked for 2 min in a solution of Ehrlich's reagent [3.2 g of *p*-(dimethylamino)-benzaldehyde in 60 mL of 95% ethanol and 60 mL of 12 N HCl], and briefly heated on a microscope slide under an open flame. Transmitted light microscopy was performed with an Axiovert 200 inverted microscope (Carl Zeiss Microimaging Inc., Thornwood, NY) at magnifications of 10×, 20×, and 40×. Axiovision version 3.0 software was used for image acquisition. Furostanol localization was visualized as dark red areas.

Purification and Confirmation of Analytical Standards. Closely following the procedure recently reported in our previous study (*28*), the steroidal glycosides **1**−**5** were isolated as analytical standards from lyophilized *L. longiflorum* bulbs (**Figure 1**). Briefly, lyophilized lily bulb powder was washed with *n*-pentane and extracted with ethanol and deionized water (7:3, v/v). After the removal of solvent, the extract was dissolved in deionized water, washed with ethyl acetate, and extracted with *n*-butanol. The organic phase was evaporated under reduced pressure and lyophilized, yielding a crude steroidal glycoside extract. The crude glycoside extract was fractionated by gel permeation chromatography and repeated semipreparative RP-HPLC to yield compounds **1**−**5**. The standards were obtained as white amorphous powders in high purity, > 98%, as determined by LC-MS and NMR.

Compound ***1***, (22*R*,25*R*)-spirosol-5-en-3*β*-yl *O*-α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranoside. ^{1}H NMR and ^{13}C NMR were consistent with the literature (*27*).

Compound ***2***, (22*R*,25*R*)-spirosol-5-en-3*β*-yl *O*-α-L-rhamnopyranosyl-(1→2)-[6-*O*-acetyl-*β*-D-glucopyranosyl-(1→4)]-*β*-D-glucopyranoside. ^{1}H NMR and ^{13}C NMR were consistent with the literature (*28*).

Compound ***3***, (25*R*)-26-*O*-(*β*-D-glucopyranosyl)furost-5-ene-3*β*,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranoside. ^{1}H NMR and ^{13}C NMR were consistent with the literature (*51*).

Compound ***4***, (25*R*)-26-*O*-(*β*-D-glucopyranosyl)furost-5-ene-3*β*,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→3)-*β*-D-glucopyranoside. ^{1}H NMR and ^{13}C NMR were consistent with the literature (*28*).

Compound ***5***, (25*R*)-26-*O*-(*β*-D-glucopyranosyl)furost-5-ene-3*β*,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-xylopyranosyl-(1→3)-*β*-D-glucopyranoside. ^{1}H NMR and ^{13}C NMR were consistent with the literature (*28*).

Nuclear Magnetic Resonance Spectroscopy (*NMR*). One-dimensional ^{1}H NMR and ^{13}C NMR spectra were acquired on an AMX-400 spectrometer and an AMX-500 spectrometer (Bruker, Rheinstetten, Germany). Samples for NMR analysis were dissolved in pyridine-d_5, and chemical shifts were calculated as δ values with reference to tetramethylsilane (TMS).

Quantitative Analysis of Steroidal Glycosides in *Lilium longiflorum*. *Sample Preparation*. Lyophilized lily organ samples were removed from the freezer and allowed to reach room temperature. The samples were ground to a fine powder with a laboratory mill (IKA Labortechnik, Staufen, Germany) and passed through a sieve (pore size = 270 mesh) (W. S. Tyler Inc., Mentor, OH). Each sample (125 mg except 250 mg for fibrous roots and fleshy roots) was weighed separately and transferred into a 50 mL volumetric flask, which was partially filled with ethanol and deionized water (7:3, v/v; 35 mL each). The samples were then extracted on a wrist-action autoshaker (45 min) (Burrell Scientific, Pittsburgh, PA), sonicated in an ultrasonic water bath (30 min) (B3500A-DTH ultrasonic bath, VWR International Inc., West Chester, PA), and filled to full volume (50 mL) with ethanol and deionized water (7:3, v/v). The solution was transferred to a centrifuge tube, centrifuged (5000 rpm for 10 min) (Sorvall RC-3C Plus, Thermo Fisher Scientific Inc.), and filtered through a 0.45 μm PTFE syringe filter (Thermo Fisher Scientific Inc.) prior to LC-MS/MS analysis.

Analytical Standard Preparation. Steroidal glycosides **1**−**5**, isolated and purified as described previously (*28*), were used as analytical standards. The analytical standards were accurately weighed into volumetric flasks (10 mL) and partially filled with ethanol and deionized water (7:3, v/v; 7 mL each). Solutions were sonicated (5 min) and filled to full volume (10 mL) with ethanol and deionized water (7:3, v/v). Solutions used for calibration curves were prepared by dilution of the stock solutions. External calibration curves were established over six data points covering a concentration range of 0.086−2.75 μg/mL for compound **1** and 0.078−2.5 μg/mL for compounds **2**−**5**. Mean areas (n = 3) generated from the standard solutions were plotted against concentration to establish calibration equations. Standard solutions were stored at 4 °C and were allowed to reach room temperature prior to analysis.

Liquid Chromatography−*Mass Spectrometry* (*LC-MS*/*MS*). LC-MS/MS analysis of *L. longiflorum* extracts was performed using an Agilent 1200 series HPLC system (Agilent Technologies Inc., Santa Clara, CA) equipped with a FC/ALS Therm autosampler thermostat, a HiP-ALS SL autosampler, a BIN Pump SL binary pump, a TCC SL thermostated column compartment, and a DAD SL diode array detector, interfaced to a 6410 triple-quadrupole LC-MS mass selective detector equipped with an API-ESI ionization source. Chromatographic separations were performed on a Prodigy C18 column (250 × 4.6 mm i.d.; 5.0 μm particle size) (Phenomenex, Torrance, CA) operated at a flow rate of 1.0 mL min^{-1}, column temperature set to 25 °C, and an injection volume of 10 μL. The binary mobile phase consisted of (A) 0.1% formic acid in deionized water and (B) 0.1% formic acid in acetonitrile. Chromatographic separations were performed using a linear gradient of 15−43% B over 40 min and then to 95% B over 5 min; thereafter, elution with 95% B was performed for 10 min. The re-equilibration time was 10 min. MassHunter Workstation Data Acquisition, Qualitative Analysis, and Quantitative Analysis software were used for data acquisition and analysis. Quantitative analysis was performed in positive ion mode. Ionization parameters included capillary voltage, 3.5 kV; nebulizer pressure, 35 psi; drying gas flow, 10.0 mL min^{-1}; and drying gas temperature, 350 °C. Full-scan mass data were collected for a mass range of m/z 100−1500. MS^2 experiments were conducted for precursor and product ion selection for steroidal glycoalkaloids **1** and **2** (**Figure 2**) and furostanol saponins **3**−**5** (**Figure 3**). Flow injection analysis (FIA) experiments were performed to optimize fragmentor voltages and collision energies. The fragmentor voltage was set at 120 V, and collision energies were set to 60, 55, 30, 25, and 25 for compounds **1**−**5**, respectively. By means of the MRM mode, the individual steroidal glycosides were analyzed using the following mass transitions: **1**, m/z 926.5 → 908.5; **2**, m/z 884.5 → 866.5; **3**, m/z 1047.5 → 885.5; **4**, m/z 1017.5 → 855.5; **5**, m/z 1017.5 → 855.5 (**Figure 4**).

Recovery. Recovery rates were calculated using the standard addition method (*52*). Lyophilized and finely ground lily organs were separately weighed, transferred into volumetric flasks (50 mL), and spiked with three different concentrations (50, 100, and 200 μg/g) of purified reference standards dissolved in ethanol and deionized water (7:3, v/v). The volumetric

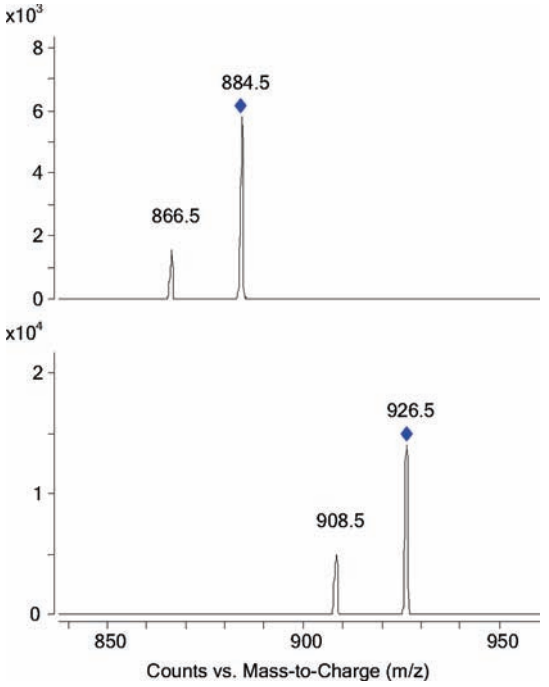

Figure 2. MS^2 product ion spectra of steroidal glycoalkaloids **1** and **2**.

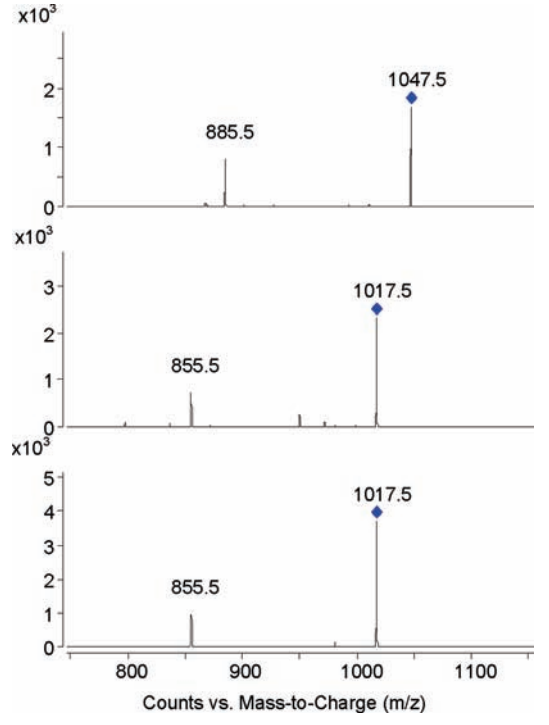

Figure 3. MS^2 product ion spectra of furostanol saponins **3**−**5**.

flasks were then partially filled with ethanol and deionized water (7:3, v/v; 35 mL each). After extraction on a laboratory shaker (45 min) and sonication (30 min), they were filled to full volume (50 mL) with ethanol and deionized water (7:3, v/v). Quantitative analysis was then performed as described above. The recovery rate for each steroidal glycoside in the different plant organs was calculated by comparing the amount of standard in the spiked sample with the content found in the lily organ sample that was not spiked with additional standards (control). Each analysis was performed in triplicate.

Thin Layer Chromatography (TLC). *L. longiflorum* bulb extract, prepared as described above, was evaporated under reduced pressure (30 °C; 1.0 × 10^{-3} bar) using a Laborota 4003 rotary evaporator (Heidolph Brinkman LLC, Elk Grove Village, IL) and lyophilized. Lyophilized bulb extract (1 mg) and compounds **3**−**5** (1 mg) were individually dissolved in methanol (0.5 mL), spotted on a 20 cm × 20 cm silica gel 60 F254 TLC plate (Merck & Co., Inc., Whitehouse Station, NJ), and developed with chloroform/methanol/water (8:4:1, v/v/v). To detect furostanols, TLC plates were developed with Ehrlich's reagent [3.2 g of *p*-(dimethylamino)benzaldehyde in 60 mL of 95% ethanol and 60 mL of 12 N HCl] and heated to 110 °C for 5 min. Bright red spots were indicative of a positive reaction.

Statistical Analysis. To examine differences in concentrations of steroidal glycosides in the different plant organs, data were subjected to analysis of variance (ANOVA) and means were separated with Fisher's protected LSD ($\alpha = 0.05$) using SAS version 9.2 for Windows (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Quantification of Steroidal Glycosides in the Different Organs of *L. longiflorum*. Recently, five steroidal glycosides including two steroidal glycoalkaloids and three furostanol saponins were identified in *L. longiflorum* (*28*). To investigate the natural distribution of these compounds in the different organs of *L. longiflorum*, compounds **1**−**5** were purified as analytical standards. To quantify compounds **1**−**5** in different organs of *L. longiflorum*, extracts prepared from bulb scales, fibrous roots, fleshy roots, leaves, lower stems, upper stems, flower buds, and mature flowers were analyzed by LC-MS/MS operating in MRM mode. To assess linearity, calibration curves were constructed over a range of six concentrations. Good linearity was achieved over the concentration ranges of 0.086−2.75 μg/mL for compound **1** and 0.078−2.50 μg/mL for compounds **2**−**5**. The correlation coefficients for compounds **1**−**5** ranged from $R^2 = 0.9997$ to $R^2 = 0.9999$. To assess the accuracy of the analytical method, recovery rates were calculated for compounds **1**−**5** in each plant organ. Standards were added in defined amounts to each plant organ sample prior to quantitative analysis and compared to a control with no standard addition. Recovery rates were calculated in the bulb scales (98.7−100.2%), fibrous roots (95.7−102.4%), fleshy roots (98.6−102.0%), leaves (95.8−103.0%), lower stems (97.7−100.1%), upper stems (95.9−102.3%), flower buds (95.3−101.0%), and mature flowers (96.3−101.9%). The recovery rates for compounds **1**−**5** in all organs analyzed were within the range of 95.7−103.0%. The precision of the method was tested by multiple injections of the same bulb scale sample ($n = 6$) and calculating the relative standard deviation (RSD) of compounds **1**−**5**. The RSD values for compounds **1**−**5** were 3.24, 2.54, 4.51, 1.63, and 2.58%, respectively. These data clearly demonstrate acceptable recovery rates, RSD, and linearity, suggesting that the LC-MS/MS method operating in MRM mode is a reliable method for the accurate quantitative determination of compounds **1**−**5** in the different organs of *L. longiflorum*.

Concentrations of compounds **1**−**5** were quantitatively determined in bulb scales, fibrous roots, fleshy roots, leaves, lower stems, upper stems, flower buds, and mature flowers of *L. longiflorum* cv. 7-8. The highest concentrations of the total five steroidal glycosides were 12.02 ± 0.36, 10.09 ± 0.23, and 9.36 ± 0.27 mg/g dry weight in flower buds, lower stems, and leaves, respectively (**Table 1**). Interestingly, the proportions of the steroidal glycoalkaloids **1** and **2** to furostanol saponins **3**−**5** were variable and decreased from the aboveground plant organs to the underground organs (**Figure 5**). The highest concentrations of the two steroidal glycoalkaloids were 8.49 ± 0.3, 6.91 ± 0.22, and 5.83 ± 0.15 mg/g dry weight in flower buds, leaves, and bulbs, respectively (**Table 1**). The two steroidal glycoalkaloids, **1** and **2**, had a similar pattern of distribution in the various organs of *L. longiflorum* (**Figure 6**). Compound **1** occurred in significantly different concentrations in all of the plant organs analyzed, except for the concentration in the bulb scale compared to

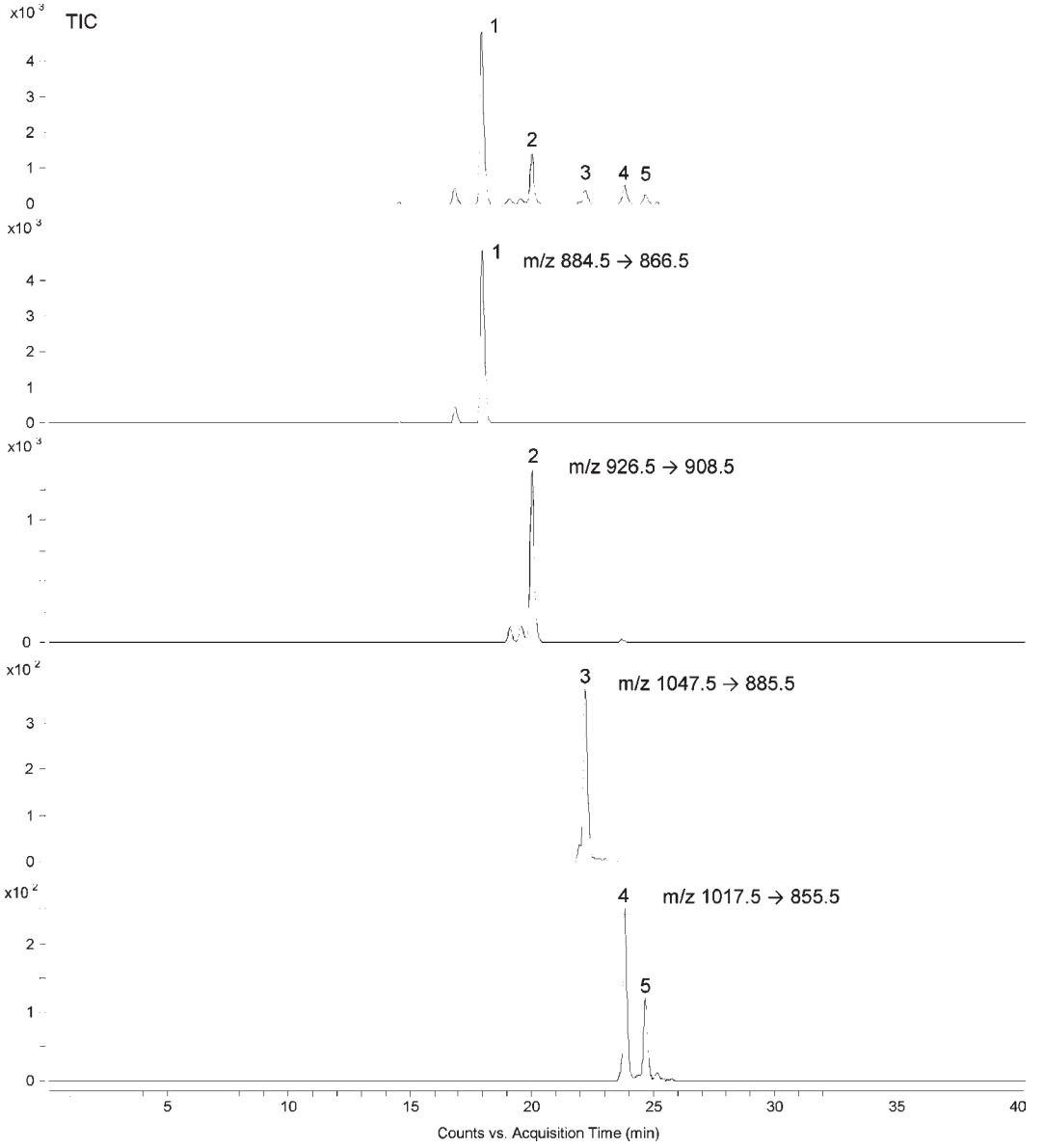

Figure 4. MS/MS chromatograms for the quantitative analysis of compounds **1**−**5** in a *L. longiflorum* bulb scale using multiple reaction monitoring (MRM) mode.

Table 1. Concentrations of Compounds **1**−**5** in the Different Organs of *L. longiflorum*

	concentration[a] (mg/g dw)							
compound	bulb scale	fibrous root	fleshy root	leaf	lower stem	upper stem	bud	flower
1	3.31c ± 0.12	0.30g ± 0.05	0.55f ± 0.02	4.23b ± 0.01	3.17c ± 0.05	2.87d ± 0.15	4.82a ± 0.19	1.96e ± 0.03
2	2.52bc ± 0.07	0.26f ± 0.04	0.44f ± 0.01	2.68b ± 0.22	2.05d ± 0.05	2.34c ± 0.12	3.67a ± 0.11	0.96e ± 0.05
total **1**−**2**	5.83c ± 0.15	0.56g ± 0.09	0.99f ± 0.01	6.91b ± 0.22	5.22d ± 0.1	5.21d ± 0.27	8.49a ± 0.30	2.92e ± 0.07
3	1.51c ± 0.08	0.47f ± 0.03	1.55c ± 0.01	2.01b ± 0.07	1.50c ± 0.12	1.15d ± 0.04	2.92a ± 0.06	0.88e ± 0.05
4	0.81c ± 0.04	0.26f ± 0.02	1.76b ± 0.04	0.29f ± 0.01	2.18a ± 0.04	0.67d ± 0.03	0.41e ± 0.01	0.35e ± 0.01
5	0.69c ± 0.05	0.13f ± 0.01	1.06b ± 0.03	0.15f ± 0.01	1.19a ± 0.02	0.37d ± 0.04	0.20e ± 0.01	0.22e ± 0.01
total **3**−**5**	3.01d ± 0.17	0.86h ± 0.07	4.37b ± 0.07	2.45e ± 0.09	4.87a ± 0.13	2.19f ± 0.05	3.53c ± 0.06	1.45g ± 0.05
total **1**−**5**	8.84d ± 0.3	1.42h ± 0.14	5.36f ± 0.08	9.36c ± 0.27	10.09b ± 0.23	7.4e ± 0.32	12.02a ± 0.36	4.38g ± 0.12

[a] Concentrations are means of triplicates ± SD, expressed on a dry weight basis (dw). Values with the same letter in each row are not significantly different ($p < 0.05$).

the lower stem, which were not significantly different from each other. Compound **1** occurred in the highest concentration of 4.82 ± 0.19 mg/g in the flower buds followed by 4.23 ± 0.01 mg/g in the leaf tissue. The concentration of **1** was significantly higher in the lower stem as compared to the upper stem, at 3.17 ± 0.05 and 2.87 ± 0.15 mg/g, respectively. In the underground organs, compound **1** occurred in the highest concentration of 3.31 ± 0.12 mg/g in the bulb scale as compared to the lowest concentrations of 0.30 ± 0.05

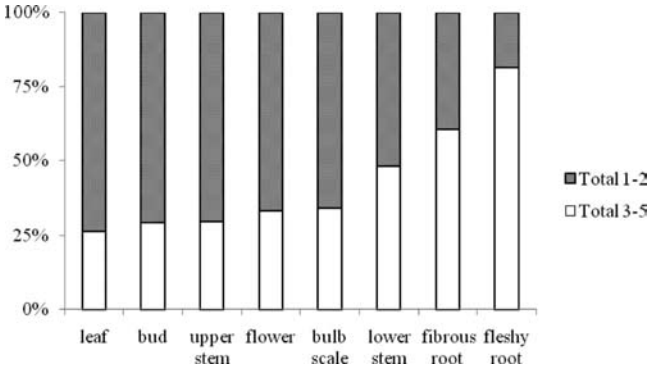

Figure 5. Proportions of steroidal glycoalkaloids **1** and **2** to furostanol saponins **3**−**5** in the different organs of *L. longiflorum*. Proportions are based on mg/g dry weight basis.

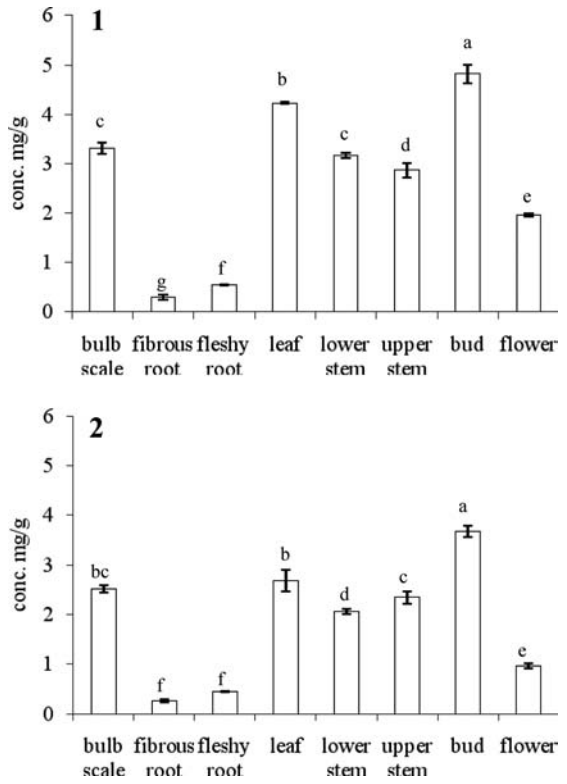

Figure 6. Concentrations of steroidal glycoalkaloids **1** and **2** in the different organs of *L. longiflorum*. Bars with the same letter are not significantly different ($p < 0.05$).

and 0.55 ± 0.02 mg/g in the fibrous roots and fleshy roots, respectively. Compound **2**, the acetylated derivative of compound **1**, was distributed similarly to compound **1**; however, it generally occurred in slightly lower concentrations. Compound **2** occurred in the highest concentration of 3.67 ± 0.11 mg/g in the flower bud followed by 2.68 ± 0.22 mg/g in the leaf tissue and 2.52 ± 0.07 mg/g in the bulb scale. In contrast to compound **1**, compound **2** was significantly higher in the upper stem as compared to the lower stem, at 2.34 ± 0.12 and 2.05 ± 0.05 mg/g, respectively. The concentration of compound **2** was not significantly different between bulb scales and leaves, 2.52 ± 0.07 and 2.68 ± 0.22 mg/g, bulbs scales and upper stem, 2.52 ± 0.07 and 2.34 ± 0.12 mg/g, and between fibrous roots and fleshy roots, 0.26 ± 0.04 and 0.44 ± 0.01 mg/g. Both steroidal glycoalkaloids occurred in the lowest concentration in the fleshy roots and fibrous roots as compared to the other organs of the plant. Interestingly, compounds **1** and **2** were lower in the flowers as compared to flower buds, which contained the highest concentration of both compounds. Similar to solanaceous plants, paired glycoalkaloids were found that differ only in the composition of the carbohydrate moiety (*24*, *31*). Although glycoalkaloids are also present in the edible parts of solanaceous plants, they can be toxic. Solanidine glycoalkaloids found in potato tubers are generally considered to be safe at concentrations of < 0.2 mg/g fresh weight (*53*). Lily bulbs contain solasodine glycoalkaloids similar to the glycoalkaloids found in eggplant, and these compounds are less toxic than the solanidine-based compounds (*31*). The content of steroidal glycoalkaloids, **1** and **2**, in lily bulbs and flower buds is > 0.2 mg/g fresh weight, but is similar to the content of solasonine and solamargine found in *Solanum macrocarpon*, the Gboma eggplant, consumed in parts of Africa, Southeast Asia, and the Caribbean. The glycoalkaloid levels in the fruits of Gboma eggplant are 5−10 times higher than the levels that are considered to be safe for human consumption based on current standards (*24*). Feeding experiments are clearly needed to determine the safe levels of *L. longiflorum* bulbs and flower buds for human consumption and if the steroidal glycoalkaloids are the toxic compounds in flowers and leaves that are responsible for poisoning in domesticated cats. Nevertheless, solasodine-based glycoalkaloids have been used to treat human skin carcinomas and are of commercial interest as a raw material for the production of pharmaceutical steroids (*46*).

The distribution of the furostanol saponins **3**−**5** was somewhat different from that of the steroidal glycoalkaoids in the various plant organs of *L. longiflorum* (**Figure 7**). The highest concentrations of the three furostanol saponins were 4.87 ± 0.13, 4.37 ± 0.07, and 3.53 ± 0.06 mg/g dry weight in lower stems, fleshy roots, and flower buds, respectively (**Table 1**). Structurally, compounds **3**−**5** are similar except for the interglycosidic linkage and terminal saccharide residues of the C-3 trisaccharide moiety (**Figure 1**). In compound **3**, the terminal sugar is a (+)-D-glucose linked from the C-1‴ carbon of the terminal sugar to the C-4′ carbon of the inner glucose. In compound **4**, the terminal sugar is (−)-L-arabinose linked from the C-1‴ carbon to the C-3′ carbon of the inner glucose. Compound **5** has the same interglycosidic linkage as compound **4**; however, it contains a (+)-(D)-xylose as the terminal sugar. Compound **3** occurred in significantly different concentrations in all of the plant organs except that the bulb scale, lower stem, and fleshy roots were not significantly different. The concentrations in the bulb scale, lower stem, and fleshy roots were 1.51 ± 0.08, 1.50 ± 0.12, and 1.55 ± 0.01 mg/g, respectively. Compound **3** occurred in the highest concentration of 2.92 ± 0.06 mg/g in the flower buds followed by 2.01 ± 0.07 mg/g in the leaf tissue. Interestingly, the fibrous roots had the lowest concentration, and similarly to the steroidal glycoalkaloids **1** and **2**, the flower buds had a higher concentration than the mature flowers.

Compounds **4** and **5** had a similar pattern of distribution in the various organs of *L. longiflorum*; however, compound **5** was slightly lower than compound **4** in all plant organs. The concentrations of both compounds **4** and **5** were not significantly different between fibrous roots and leaves or between flower buds and mature flowers. Interestingly, in contrast to the other compounds, compounds **4** and **5** occurred in the highest concentration in the fleshy roots and occurred in lower concentrations in the leaf tissue, flower buds, and mature flowers. The differences in distribution of compounds **4** and **5** as compared to compound **3** may be due to differences in the carbohydrate moiety. Compounds **4** and **5** both contain a pentose as the terminal sugar linked via the C-3′ carbon of the inner glucose, whereas compound **3** contains a hexose linked via the C-4′ carbon of the inner glucose. Further studies involving site of biosynthesis, subcellular

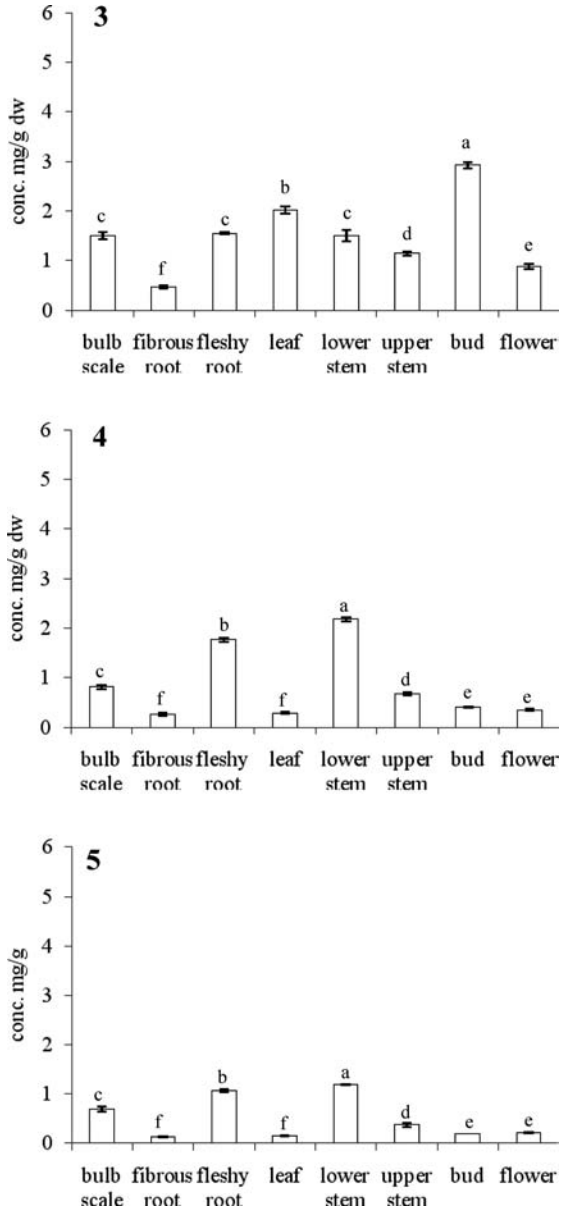

Figure 7. Concentrations of furostanol saponins **3**−**5** in the different organs of *L. longiflorum*. Bars with the same letter are not significantly different ($p < 0.05$).

localization, and transport would have to be conducted to support this hypothesis.

Histological Visualization of Furostanol Localization in Bulb Scale Sections of *L. longiflorum*. The Ehrlich reagent color reaction was employed to visualize furostanols in bulb scale sections. The furostanols gave a bright red positive reaction, whereas the steroidal glycoalkaloids were not positive for the reaction, substantiated by the staining of reference compounds that were separated by TLC. Additionally, a crude extract of bulb scales was separated by TLC, and only furostanol bands gave a positive reaction, suggesting that staining bulb scales with Ehrlich reagent should not produce positive reactions with nontarget compounds. Macroscopically, bulb scale cross sections show accumulation of furostanols in the outermost layers of the bulb scale. Additionally, a positive reaction was observed surrounding three vascular bundles located in the mesophyll, suggesting furostanol localization is associated with vascular bundles and closely adjacent cells (**Figure 8**). Interestingly, a positive reaction was not observed in the mesophyll, suggesting preferential accumulation and elevated

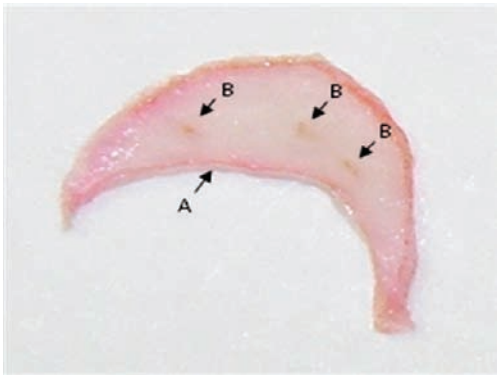

Figure 8. Histochemical staining of a bulb scale section. Arrows indicate the epidermis (A) and three vascular bundles (B). Red color indicates the presence of furostanols.

levels of furostanols in the outermost layer of the bulb scale and association with vascular bundles. Microscopically, furostanols were visualized in the highest intensity within the cells of the basal plate, and vascular bundles and preferential accumulation in the intercellular spaces between the mesophyll cells and palisade cell layer were observed (**Figure 9**). These observations are consistent with a histochemical analysis of mature leaf slices of *Achyranthus bidentata* that showed triterpenoid saponin accumulation in palisade tissue and phloem cells of the main vein vascular bundles and in phloem cells of the normal and medullary vascular bundles of stem sections (*54*). Interestingly, in oats, *A. sativa*, the fluorescent steroidal saponin, avenacin A-1, has been visualized under UV light to be localized in the epidermal cell layer of roots (*38*). Similarly, in a histological study of *Dioscorea caucasia*, furostanol accumulation was observed in specialized idioblasts in the upper and lower leaf epidermis and was not observed in the leaf mesophyll (*50*). Consistent with the observations in *D. caucasia* and *A. bidentata*, there was no positive staining reaction in the mesophyll of the bulb scale of *L. longiflorum*, suggesting lower levels of furostanols in these tissues.

Quantification of Steroidal Glycosides within Bulb Scales of *L. longiflorum*. LC-MS/MS operating in MRM mode was employed to quantitatively determine the levels of compounds **1**−**5** within whole bulb scales, the inner portion of bulb scales, and the outermost portion of bulb scales. The outermost layers of intact lyophilized bulb scales were carefully excised with a scalpel. The excised outermost cell layer was approximately 15% of the average mass of the whole intact bulb scale. Quantitative analysis was performed on whole bulb scale, the outermost layer of bulb scale (mostly epidermal and subepidermal cells), and the innermost layer of bulb scale (mostly mesophyll and vascular bundles) (**Table 2**). Consistent with what was observed from the histological experiment, concentrations of furostanols **3**−**5** were higher in the outermost portion of the bulb scale verses the inner bulb scale tissue. These quantitative data confirm the qualitative histological observations made for localization of furostanols in bulb scale sections. Interestingly, the steroidal glycoalkaloids **1** and **2** had the same organ distribution pattern as the furostanols and occurred in elevated levels in the outermost portion of the bulb. The relative portions of compounds **1**−**5** in the outermost layer of bulb scales were 38, 31, 20, 6, and 5%, respectively (**Figure 10**). The relative portions of compounds **1**−**5** in the innermost portion of bulb scales were 9, 12, 17, 34, and 28%, respectively. The relative proportions of compounds **1**−**5** in the outermost layer of the bulb scale are similar to that of the whole bulb scale. The relative proportions of compounds **1**−**5** in the

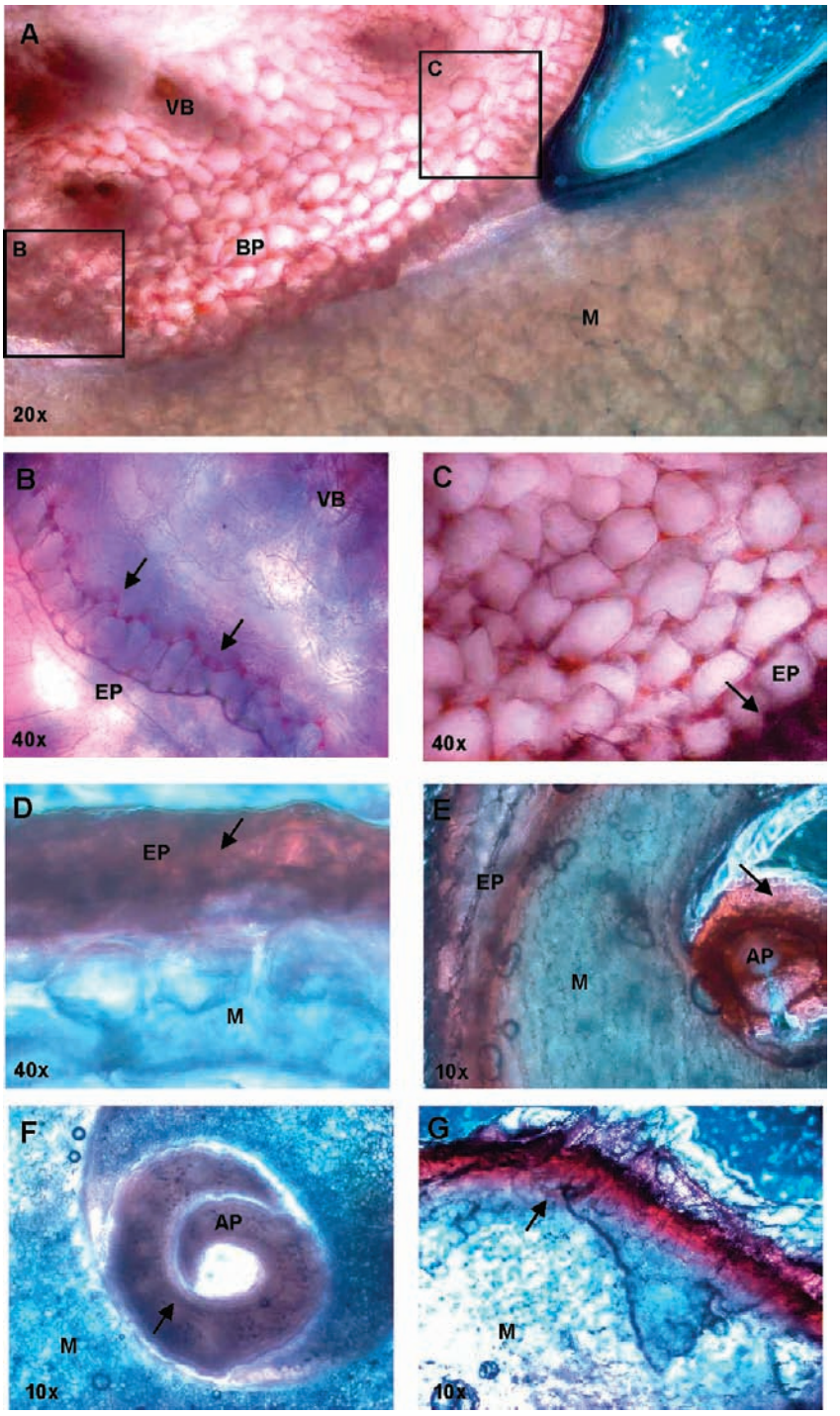

Figure 9. Histochemical analysis of bulb basal plate and bulb scale sections: (**A**) Bulb basal plate and adjacent bulb scale section (lettered boxes indicate topography of images **B** and **C**); (**B**) subepidermal intercellular furostanol accumulation along the palisade parenchyma of the basal plate (red); (**C**) intercellular furostanol accumulation between spongy tissue cells and palisade parenchyma of the basal plate (red); (**D**) epidermal (red) and mesophyll cells of a bulb scale section; (**E**) bulb scale epidermis (red), mesophyll and apical meristem (red); (**F**) apical meristerm (red) and mesophyll cells; (**G**) bulb scale epidermal cells (red) and mesophyll cells. Furostanol localization is visualized as dark red areas. VB, vascular bundles; BP, basal plate; M, bulb scale mesophyll; EP, epidermal cells; AP, apical meristem.

innermost section of the bulb scale are most similar to those of the fleshy roots, suggesting that the vascular bundles are most likely contributing to the elevated levels of compound **3**−**5** in this tissue, which is consistent with the histological visualizations (**Figure 11**). In summary, the outermost cell layer of bulb scales that are associated with the bulb epidermis had elevated levels of both steroidal glycoalkaloids and furostanols. The innermost section of bulb scales had lower levels; however, the proportions of furostanols to steroidal glycoalkaloids were different, demonstrating that the cells associated with the vascular bundles have proportions of compounds **1**−**5** similar to those of the fleshy roots as compared to the bulb epidermis. Elevated levels and preferential accumulation of steroidal glycosides in the outermost cell layer of bulb scales and the cells associated with vascular bundles may play a role in wound response and plant−pathogen interaction of *L. longiflorum*.

Table 2. Concentrations of Compounds **1**−**5** in Whole Bulb Scale, Bulb Epidermis, and Bulb Mesophyll

	concentration[a] (mg/g dw)		
compound	whole bulb scale	bulb epidermis	bulb mesophyll
1	3.31b ± 0.12	13.76a ± 0.5	0.06c ± 0.01
2	2.52b ± 0.07	11.22a ± 0.3	0.07c ± 0.01
3	1.51b ± 0.08	7.27a ± 0.4	0.10c ± 0.01
4	0.81b ± 0.04	1.91a ± 0.1	0.20c ± 0.02
5	0.69b ± 0.05	1.90a ± 0.1	0.17c ± 0.02

[a] Concentrations are means of triplicates ± SD, expressed on a dry weight basis (dw). Values with the same letter in each row are not significantly different ($p < 0.05$).

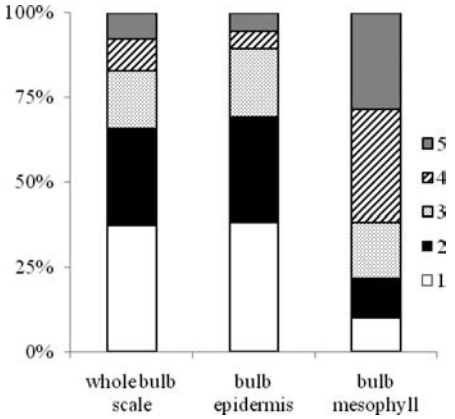

Figure 10. Proportions of compounds **1**−**5** in whole bulb scale, bulb epidermis, and bulb mesophyll. Proportions are based on mg/g dry weight basis.

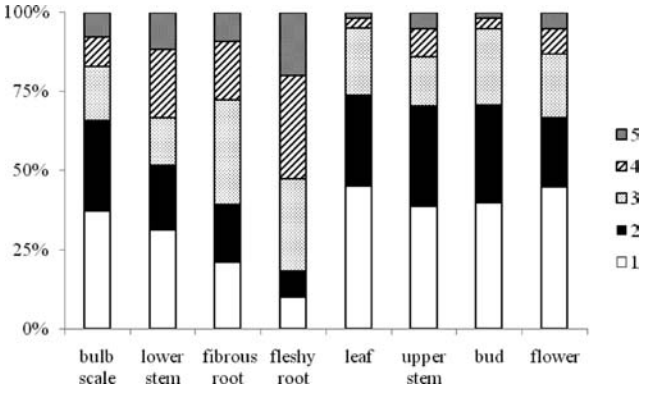

Figure 11. Proportions of compounds **1**−**5** in different organs of *L. longiflorum*. Proportions are based on mg/g dry weight basis.

In the present work, two steroidal glycoalkaloids, (22*R*,25*R*)-spirosol-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside and (22*R*,25*R*)-spirosol-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-[6-*O*-acetyl-β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside, and three furostanol saponins, (25*R*)-26-*O*-(β-D-glucopyranosyl)furost-5-ene-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside, (25*R*)-26-*O*-(β-D-glucopyranosyl)furost-5-ene-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→3)-β-D-glucopyranoside, and (25*R*)-26-*O*-(β-D-glucopyranosyl)furost-5-ene-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-xylopyranosyl-(1→3)-β-D-glucopyranoside, were quantified in the different organs of *L. longiflorum* for the first time. The highest concentrations of steroidal glycosides were detected in flower buds, lower stems, and leaves. The steroidal glycoalkaloids were detected in higher concentrations as compared to the furostanol saponins in all of the plant organs except for the fibrous and fleshy roots. The proportions of steroidal glycoalkaloids to furostanol saponins were higher in the plant organs exposed to light and decreased from the aboveground organs to the underground organs. The highest concentrations of the steroidal glycoalkaloids were detected in flower buds, leaves, and bulbs. Both steroidal glycoalkaloids had a similar pattern of distribution in the various plant organs; however, the acetylated derivative occurred at lower levels. The furostanol saponins were detected in the highest concentrations in the lower stems, fleshy roots, and flower buds. Interestingly, the flower buds contained the highest concentrations of compounds **1**−**3**, and the fleshy roots contained the highest levels of compounds **4** and **5**. In addition, differential accumulation of steroidal glycosides was observed in the basal plate, bulb scale epidermal cells, and vascular bundles. Quantitative analysis of steroidal glycosides in the different organs of *L. longiflorum* is the first step to developing insight into the biological role these compounds play in plant metabolism, plant development, and plant−pathogen interactions. The results of this study will aid in the development of future studies in animal and human health and toxicology and of commercial applications such as functional foods, cosmetics, and pharmaceuticals.

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